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Review Article

Multilayered control of chromosome replication in *Caulobacter crescentus*

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The environmental *Alphaproteobacterium Caulobacter crescentus* is a classical model to study the regulation of the bacterial cell cycle. It divides asymmetrically, giving a stalked cell that immediately enters S phase and a swarmer cell that stays in the G1 phase until it differentiates into a stalked cell. Its genome consists in a single circular chromosome whose replication is tightly regulated so that it happens only in stalked cells and only once *per* cell cycle. Imbalances in chromosomal copy numbers are the most often highly deleterious, if not lethal. This review highlights recent discoveries on pathways that control chromosome replication when *Caulobacter* is exposed to optimal or less optimal growth conditions. Most of these pathways target two proteins that bind directly onto the chromosomal origin: the highly conserved DnaA initiator of DNA replication and the CtrA response regulator that is found in most *Alphaproteobacteria*. The concerted inactivation and proteolysis of CtrA during the swarmer-to-stalked cell transition license cells to enter S phase, while a replisome-associated Regulated Inactivation and proteolysis of DnaA (RIDA) process ensures that initiation starts only once *per* cell cycle. When *Caulobacter* is stressed, it turns on control systems that delay the G1-to-S phase transition or the elongation of DNA replication, most probably increasing its fitness and adaptation capacities.

Introduction

DNA replication is controlled with exquisite precision in all cell types to ensure that each daughter cell inherits one copy of complete chromosome(s) following each division event. Control mechanisms largely focus on the initiation step of the replication process, when the replisome is assembled onto DNA. In nearly all bacteria, the highly conserved initiator of chromosome replication is DnaA [1,2]. It typically binds to several DnaA boxes located on the chromosomal origin and oligomerizes to open the DNA double helix at an AT-rich region within the origin. It also interacts with helicases to load them onto the DNA. Subsequently, the replicative DNA polymerase and its β-sliding clamp (DnaN) are recruited onto leading and lagging strands to initiate bi-directional and processive DNA replication. The proper timing of chromosome replication is highly dependent on controlling the levels, the activity and the availability of DnaA in bacteria. The analysis of a variety of bacteria now reveals to which extent control mechanisms are conserved, or not, in different bacterial classes or species [3,4]. It also shows that bacteria often control the DNA replication process to modulate their proliferation in response to environmental cues directly connected to their biological niche, through the regulation of DnaA or of other more specific regulators.

Caulobacter crescentus (henceforth *Caulobacter*) is an aquatic *Alphaproteobacterium* that emerged as a powerful model system to study the regulation of the bacterial cell cycle. This bacterium divides asymmetrically at the end of each cell cycle giving daughter cells with distinct developmental and replicative fates (Figure 1) [5]. The first one is a replication incompetent (G1-phase) swarmer cell, while the second one is a replication competent (S-phase) stalked cell. To initiate the replication of its unique circular chromosome, the swarmer cell must first differentiate into a stalked cell. This relatively slow growing bacterium, compared with the most studied *Escherichia coli* model system, never displays

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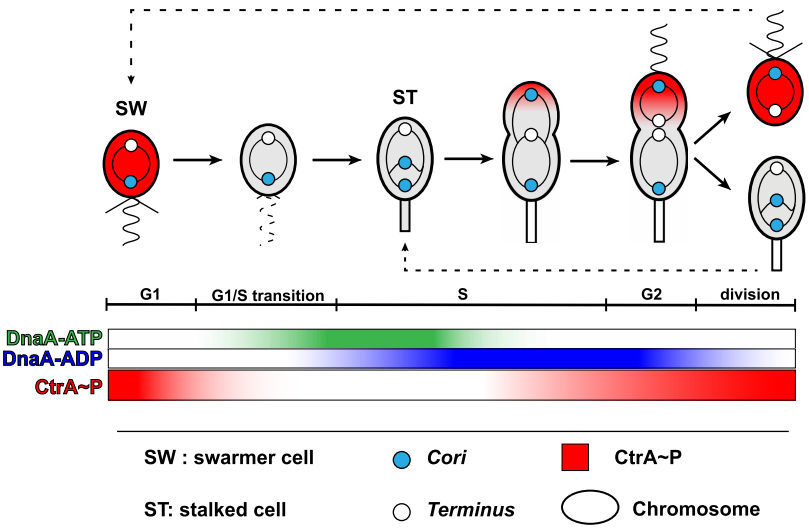

Figure 1. Graphical representation of the *Caulobacter* cell cycle and of the abundancy of the DnaA and CtrA main regulators of the initiation of chromosomal replication.

In swarmer cells, the initiation of chromosomal replication is inhibited by high levels of CtrA~P (red color) binding to the chromosomal origin of replication (*Cori*, blue circle). During the swarmer-to-stalked cell transition, CtrA~P is proteolyzed at the cell pole where the stalk will be built. Coincidentally, DnaA is synthesized and binds to ATP. DnaA-ATP (green color) binds to the *Cori* and is active to initiate replication. As soon as DNA replication has started, HdaA is recruited to the replisome and activated to stimulate to conversion of DnaA-ATP into DnaA-ADP (blue color) and DnaA proteolysis. This Regulated Inactivation of DnaA (RIDA) process prevents re-initiation during the same cell cycle. In early predivisional cells, CtrA is re-synthesized and phosphorylated, but gets efficiently dephosphorylated and proteolyzed in the swarmer cell compartment of late pre-divisional cells to inhibit the initiation of DNA replication in the swarmer progeny. Although this was not directly demonstrated, CtrA~P may form a gradient in pre-divisional cells, being the most abundant at the flagellated cell pole even before cell compartmentalization, due to polarized upstream regulators of CtrA phosphorylation and degradation [83].

more than two replication forks at work within the same cell and over-initiation events are severely deleterious [6]. It is relatively easy to isolate nearly pure populations of swarmer cells from mixed populations of *Caulobacter* cells, facilitating studies on the regulation of the timing of DNA replication during the bacterial cell cycle.

This review focuses on recently discovered mechanisms that control the replication of the chromosome of *Caulobacter* cultivated in favorable or less-favorable growth conditions, with particular emphasis on the regulation of the DnaA initiator and of the CtrA inhibitor of chromosome replication that are both highly conserved in *Alphaproteobacteria*.

The origin of replication of the *Caulobacter crescentus* chromosome

The origin of replication of the *Caulobacter* chromosome (*Cori*) was mapped years ago at a chromosomal locus located close to the *hemE* and overlapping the *CCNA_00001* (*duf299*) open reading frames (ORF) (Figure 2) [6–8]. It carries DNA motifs with affinity for several known regulators of the *Caulobacter* cell cycle, including the initiator DnaA, the response regulator CtrA, the nucleoid associated proteins IHF (Integration host Factor) and GapR, and the DNA methyltransferase (MTase) CcrM [6,7,9]. As assumed in nearly all bacterial species, DnaA, and several of the DnaA boxes found on the origin, are indispensable for the initiation of chromosome replication in *Caulobacter* [7,10]. DnaA binds to seven DnaA boxes on the *Cori*; five of these are low-affinity W-boxes and two are moderate-affinity G-boxes (Figure 2) [7]. Although one of the W-box overlaps with a DNA motif that can be methylated by the DNA MTase CcrM (Figure 2), it appears that methylation of the *Cori* by CcrM is not required for DNA replication in *Caulobacter,* since the chromosome content of Δ*ccrM* cells is identical with that of wild-type cells [11,12]. Also, consistent with this finding, a homolog of the

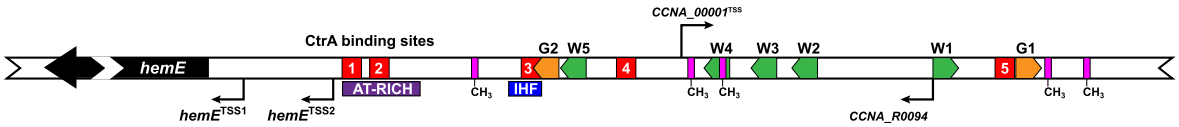

Figure 2. Organization of the *Caulobacter* chromosomal origin of replication (*Cori*).

The *Cori* carries two classes of DnaA binding sites: G-boxes (G1 and G2; orange arrows) of moderate affinity and W-boxes (W1–W5; green arrows) of low affinity. Note that the G2 box overlaps one of the CtrA binding sites (red squares), suggesting that DnaA-ATP may compete with CtrA when binding to the *Cori*. The *Cori* also carries an IHF binding site (blue square) and five GANTC sites (fuchsia color; $-CH_3$) that are methylated by the CcrM DNA methyltransferase. IHF may promote the opening of the DNA double helix at the AT-rich region (purple box) when DnaA-ATP is bound to the *Cori*. In addition, the transcriptional start sites (TSS; represented by a thin black arrow) for three RNAs are located in the *Cori*: the *duf299 (CCNA_00001)* and *hemE* mRNAs and the *CCNA_R0094* small non-coding RNA. Transcription starting at these TSS might influence initiation.

methylation-dependent DNA binding protein SeqA, which controls replication initiation in *E. coli* [3], cannot be found in the *Caulobacter* proteome. The relatively low affinity of DnaA for the *Cori* DnaA boxes might be connected with the existence of CtrA, which inhibits the initiation, supposedly through a direct competition with DnaA when binding to the *Cori*. Indeed, five CtrA binding sites are found on the *Cori* and one of them overlaps one of the two moderate-affinity DnaA G-boxes (Figure 2). The *ctrA* gene is essential for the survival of *Caulobacter* and partial loss of CtrA function leads to premature initiation of DNA replication in swarmer cells [13,14]. Notably, an IHF binding site overlaps one of the CtrA binding sites (Figure 2) [15], indicating that IHF may promote the disassembly of CtrA on the *Cori* during the swarmer-to-stalked cell transition [16]. Targeted mutagenesis experiments eliminating the CtrA binding sites on the *Cori*, however, showed that the inhibition of DNA replication by CtrA is dispensible when *Caulobacter* is cultivated in minimal medium [17]. In addition to DnaA, CtrA and IHF, the GapR protein shows affinity for *Cori* [9]. GapR is a newly discovered and conserved nucleoid associated protein that appears as critical for chromosome replication and growth in *Caulobacter* [9,18,19], most probably by promoting the ability of topoisomerases to relax positive supercoils that accumulate ahead of the replication fork [20]. Considering that GapR is more abundant in stalked than in swarmer cells [9], it might also promote the binding of DnaA to the *Cori*, although this was not tested directly. Finally, the presence of two ORFs [21] and of a gene transcribed into a small non-coding RNA [22,23] in the *Cori* region (Figure 2) suggests that the transcription of these elements could potentially interfere with or promote the opening of the DNA double helix for initiation. Consistent with this possibility, early findings showed that transcription from one of the *hemE* promoters is required for chromosome replication in *Caulobacter* [24].

CtrA restricts the initiation of chromosome replication to stalked cells

CtrA is a conserved response regulator, which is found in most *Alphaproteobacteria* [25]. It needs to be phosphorylated to be active and to inhibit the initiation of DNA replication [13]. Logically, very strict regulatory mechanisms control the levels of active CtrA~P (Figures 1 and 3A), to ensure that chromosome replication can still start once per cell cycle and specifically during the swarmer-to-stalked cell transition of *Caulobacter* or in the stalked progeny following cell division. CtrA~P is very abundant in swarmer cells, where it binds to the *Cori* with high affinity to maintain cells in G1 phase, and in pre-divisional cells, where it plays essential roles in the regulation of gene expression (Figure 1) [26,27]. Following cytoplasmic compartmentalization in late pre-divisional cell, the levels of CtrA become highly dissimilar: the flagellated compartment of the cell contains ~22,000 molecules of CtrA [28], while the stalked compartment contains undetectable levels of CtrA. These strong temporal and spatial variations are mostly dependent on the activity of the essential CckA histidine kinase/phosphatase, which is at the top of a phosphorelay controlling CtrA phosphorylation and proteolysis (Figure 3A) [5,6]. Indeed, although *ctrA* transcription varies significantly during the cell cycle, due to tight control by the GcrA epigenetic regulator [29,30], by the CtrA-associated SciP regulator [31,32] and by self-regulation [33], this temporal regulation is not required for the control of the initiation of replication since the constitutive transcription of *ctrA* does not lead to replication defects [26]. Still, sufficient transcription of *ctrA*

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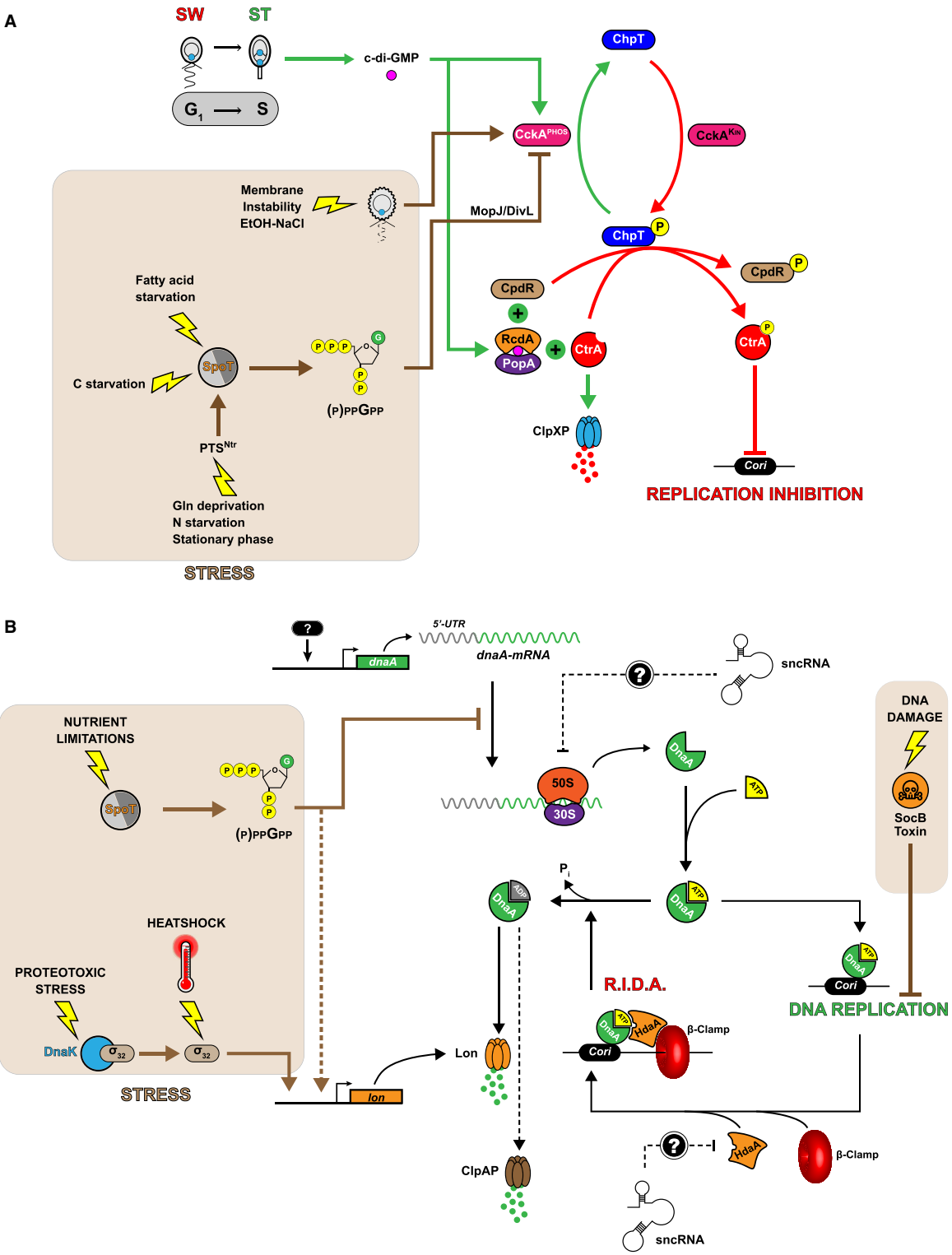

Figure 3. Graphical representation of the regulatory circuits controlling CtrA and DnaA activity in *Caulobacter* cells cultivated in optimal (no background color) or stressful (brown background color) growth conditions. Part 1 of 2

(A) Model for the regulatory network controlling the levels and the activity of CtrA. During the swarmer-to-stalked cell transition, the activity of CckA switches from a kinase ($CckA^{KIN}$; red arrows) to a phosphatase ($CckA^{PHOS}$; green arrows), reducing the levels of ChpT~P. In turn, CtrA~P can no more accumulate and gets actively degraded by a CpdR- and PopA/RcdA-dependent ClpXP proteolysis complex, triggering the initiation of DNA replication by DnaA-ATP. When nutrient levels

Figure 3. Graphical representation of the regulatory circuits controlling CtrA and DnaA activity in *Caulobacter* cells cultivated in optimal (no background color) or stressful (brown background color) growth conditions. Part 2 of 2

become too limiting, the kinase activity of CckA is probably stimulated in a (p)ppGpp- and MopJ-dependent manner to delay the G1-to-S phase transition. In contrast, membrane stresses appear to promote the phosphatase activity of CckA and a reduction in CtrA∼P levels, maybe to delay cell division without inhibiting chromosome replication. **(B)** Model of the regulatory network controlling the levels and the activity of DnaA. Newly synthesized DnaA associates with ATP, which can then initiate DNA replication once CtrA∼P levels are low enough. Immediately after replication has started active DnaA-ATP is converted into inactive DnaA-ADP by the β-clamp binding HdaA protein. This conversion also appears to promote the degradation of DnaA by the Lon protease. This Regulated Inactivation and proteolysis of DnaA (RIDA) process ensures that DNA replication cannot re-initiate before the next cell cycle. Another β-clamp binding protein named SocB can inhibit the elongation of DNA replication in response to DNA damage. When *Caulobacter* is exposed to stresses such as nutrient limitations or proteotoxic stresses, DnaA levels drop due to an inhibition of *dnaA* translation and/or to a stimulation of DnaA proteolysis.

is needed for the correct control of replication initiation, since a loss of CtrA activity leads to severe replication defects and cell death [13].

CckA is the most important regulator of CtrA levels and activity. It acts as a kinase phosphorylating the ChpT phosphotransferase in swarmer and pre-divisional cells, while it acts as a phosphatase in stalked cells (Figure 3A). In turn, phosphorylated ChpT transfers its phosphate group to the two response regulators CtrA and CpdR, activating and stabilizing CtrA [34,35]. The switch in CckA activity from a kinase to a phosphatase mode is controlled by the cyclic diguanylate (c-di-GMP) second messenger (Figure 3A) [36,37], whose levels rise specifically during the swarmer-to-stalked cell transition and in the swarmer compartment of late pre-divisional cells [38] and which interacts directly with one of the two PAS domains of CckA [39]. In addition, this PAS domain interacts with the DivL pseudokinase in complex with the DivK response regulator to further control the activity of CckA [40–42]. The kinase-to-phosphatase switch in CckA activity dictates when the initiation of chromosome replication can start, so that it takes place specifically during the swarmer-to-stalked cell transition or in the newborn stalked cell following division [14]. In addition to inactivating CtrA by de-phosphorylation, this switch also promotes CtrA degradation by the ClpXP protease by de-phosphorylating CpdR (Figure 3A) [34,35]. When CpdR is un-phosphorylated, it associates with ClpX and with a complex composed of the c-di-GMP-stimulated PopA protein and of the RcdA adaptor to turn on CtrA proteolysis at the cell pole [43–47]. Although CtrA degradation takes place at the stalked pole of wild-type cells, recent findings, however, suggest that it can take place even more efficiently in the cytoplasm of cells lacking the PopZ cell pole organizer [48,49]. Once the replication of the chromosome is licensed to start through the strong reduction in CtrA∼P levels, DnaA can bind to the *Cori* to initiate DNA replication.

DnaA controls the frequency of the initiation of chromosome replication

Control of the abundancy of active DnaA ensures that replication can start once, but only once, *per* cell cycle [6,50]. Levels must be high enough in stalked cells, but shut down right after replication initiation to prevent over-initiation events in stalked and early pre-divisional cells, when CtrA∼P levels are minimal.

A first level of regulation is through the control of *dnaA* transcription that peaks right before the initiation of chromosome replication during the swarmer-to-stalked cell transition [6,51]. Mechanisms controlling *dnaA* transcription are still unclear although the analysis of *dnaA* promoter elements indicates that the efficiency and the timing of *dnaA* transcription may be controlled by a transcriptional activator binding close to a GAGTC motif upstream of the −35 region [52,53]. A second level of regulation is through the inhibition of *dnaA* translation by a long untranslated region (5'UTR) upstream of the translational start codon (Figure 3B), but this mechanism does not appear to control the timing of *dnaA* expression [53]. Importantly, the transcriptional and post-transcriptional controls of DnaA synthesis play only a marginal role in controlling when, and at which frequency, DNA replication can initiate during the *Caulobacter* cell cycle, since the artificial and constitutive expression of *dnaA* does not lead to replication defects in *Caulobacter*. Instead, these mechanisms may be more useful to control DnaA levels in response to environmental signals, rather than to cell cycle cues.

The most important mechanism controlling the frequency of replication initiation is the so-called Regulated Inactivation of DnaA (RIDA) process that appears as essential for the survival of *Caulobacter* [14,54,55]. This

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process restricts the levels of active DnaA by stimulating its ATPase activity, leading to the concerted inactivation and degradation of DnaA (Figure 3B) [6,50]. There are now clear indications that DnaA needs to be associated with ATP to initiate DNA replication [14,54,55], as it is also the case in many other bacterial species [3]. The RIDA process takes place right after the initiation of DNA replication, through a stimulation of the ATPase activity of DnaA by the HdaA protein, preventing lethal over-initiation events. The current model is that HdaA interacts with the β-sliding clamp of the replisome once it is loaded onto the DNA at the onset of DNA replication and that this interaction switches on the activity of HdaA and the subsequent RIDA process [54,56]. In addition, the conversion of DnaA-ATP into DnaA-ADP during the RIDA process leads to a significant destabilization of DnaA at the onset of DNA replication during the swarmer-to-stalked cell transition [57,58]. Two different ATP-dependent proteases can recognize DnaA in *Caulobacter* (Figure 3B). The first one is ClpAP, which appears to destabilize DnaA independently of the nucleotide bound to it and preferentially under stress conditions, since the ClpS adaptor inhibits this pathway in exponentially growing cells [59]. The second one is Lon [60] and seems to have a preference for DnaA-ADP rather than DnaA-ATP [59], suggesting that it preferentially degrades DnaA during the S phase of the cell cycle when the RIDA process is active. Thus, Lon probably contributes to controlling the intracellular levels of active DnaA as a function of the cell cycle to prevent over-initiation events. As described below, it also plays a key role in adjusting DnaA levels and regulating DNA replication in response to stresses.

Control of chromosome replication during non-optimal growth conditions

Bacteria must coordinate the replication of their genome with their growth rate. This is all the more important for bacteria that live in oligotrophic environments, like *Caulobacter*, and that are thus frequently exposed to nutrient limitations and environmental stresses.

When exposed to nutrient limitations, *Caulobacter* turns on a so-called stringent response that targets DnaA and CtrA to slow down or arrest the replication of its chromosome. This response is based on the production of guanosine tetra- or penta-phosphate ((p)ppGpp) by the SpoT enzyme [61], in response to carbon or nitrogen starvation [62] or in response to fatty acid depletion (Figure 3A) [63]. Recent findings also showed that SpoT is specifically activated by elements of the nitrogen-related phosphotransferase system (PTS^{Ntr}) in response to low glutamine levels [64,65]. The (p)ppGpp alarmone then appears to inhibit DnaA synthesis (Figure 3B) and CtrA degradation (Figure 3A), leading to a severe inhibition of the initiation of chromosome replication [66]. The stabilization of CtrA by (p)ppGpp appears to be dependent on the up-regulation of the conserved MopJ regulator, which targets the DivL-dependent pathway controlling CckA activity (Figure 3A), although this may not be the only pathway involved [67]. The complex regulatory system controlling DnaA levels in response to nutrient limitations appears to include an inhibition of *dnaA* translation (Figure 3B), leading to a rapid clearance of DnaA by the Lon protease [68]. Interestingly, this translational inhibition also takes place in starved Δ*spoT* mutant cells, indicating that other unknown (p)ppGpp-independent pathways are also involved in the regulation of DNA replication in response to low nutrient levels [68].

When exposed to DNA/protein damaging conditions, *Caulobacter* also stops or slows down the replication of its chromosome. Two such regulatory pathways have now been identified (Figure 3B). The first one is based on the detection of unfolded or damaged proteins by the DnaK chaperone, which leads to a stabilization of the σ^{32} heat-shock factor and the consequent activation of *lon* transcription and Lon-mediated degradation of DnaA [60]. Thus, proteotoxic and severe heat-shock stresses block the initiation of DNA replication by DnaA [60,69]. The second one is based on the stabilization of the SocB toxin in response to DNA damage. In turn, SocB can bind to the β-clamp of the DNA polymerase to inhibit the elongation of DNA replication, most probably through a disassembly of the replicative DNA polymerase complex [70].

Surprisingly, *Caulobacter* adopts a rather opposite strategy when facing stress conditions affecting the integrity of its membrane, such as exposure to ethanol or high salt concentrations in its environment or exposure to mild heat-shocks. Indeed, these stresses promote the phosphatase activity of CckA in a DivL- and c-di-GMP-independent manner, leading a rapid decrease in the levels of active CtrA∼P [71]. As a consequence, fewer stressed cells are in G1 phase and cells appear longer with an abnormally high DNA content compared with non-stressed cells. Future work should aim at understanding why the inhibition of CtrA activity provides an advantage to cells facing membrane stresses, while it appears to be deleterious to cells facing nutritional stresses.

Perspectives

This review aimed at summarizing the current view on the control of DNA replication in *Caulobacter*. Most, although not all, of the pathways regulating chromosome replication target the DnaA initiator or the CtrA response regulator. In fast growing conditions, the tight regulation of their activity is mostly dependent on the RIDA process and on the activity of the CckA kinase/phosphatase. A common point between the RIDA process and the CckA phosphatase is that they promote the inactivation and the proteolysis of their targets (Figure 3). Interestingly, the (p)ppGpp alarmone affects CtrA and DnaA to adjust the length of the G1 phase under fast-growing conditions [72], or to block cells in the G1 phase under stressful conditions [64,65,67]. DnaA synthesis also appears to be regulated by (p)ppGpp and growth conditions [66,68], but mechanisms involved in these control systems are still ill-defined. Interestingly, experimental and computational evidences suggest that up to 10 small non-coding RNAs (sncRNAs) might target the *dnaA* and *hdaA* messenger RNAs (Figure 3B) [22,23,73]. Considering that the expression of several of these is cell cycle-regulated or regulated in response to stresses [22,23,74,75], and that the Hfq RNA chaperone is required for metabolic homeostasis [76], it is tempting to predict that translational regulation by sncRNAs might also play an important role in fine-tuning the levels and the activity of DnaA (Figure 3B). Other unknown pathways are probably involved in adjusting DNA replication with cell growth, most probably by connecting the central metabolism with DNA replication. A recent study, for example, raised the possibility that a member of the Lrp family of transcription factors, named PutR, might connect proline intracellular levels with the elongation of DNA replication and cell division in *Caulobacter* [77].

It is noteworthy that DnaA and CtrA are also important transcription factors [27,78]. The impact of the RIDA process on the control of DnaA-regulated genes has not been investigated in detail, but preliminary findings suggest that the nucleotide binding to DnaA might also influence the transcription of a subset of these genes and chromosome segregation [55,79]. This might be used by *Caulobacter* to coordinate DNA replication with other processes required for cell cycle progression.

Finally, it is also interesting to mention that while the regulation of DnaA appears to be a widespread mechanism used by most bacteria to control chromosome replication, the involvement of CtrA in replication control is restricted to only a subset of them. Indeed, although CtrA is well conserved in *Alphaproteobacteria*, its regulon appears to evolve rapidly [80–82]. Then, determining whether DNA replication is under negative control in other *Alphaproteobacteria* and understanding the associated mechanisms could provide interesting new information on the evolution of complex networks controlling chromosome replication in bacteria.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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